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## Thermal tolerance and heat shock proteins in encysted embryos of *Artemia* from widely different thermal habitats

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### Abstract

Encysted embryos (cysts) of the brine shrimp, *Artemia* provide an excellent model system for the study of biochemical adaptation to environmental extremes. Here, we describe an experiment in which cysts of *A. franciscana* from the San Francisco Bay (SFB), California, U.S.A., were inoculated into experimental ponds in the Mekong Delta region of Vietnam where water temperatures are much higher than the SFB. Cysts produced in each of three successive growing seasons (1996–1998) were collected and examined in the laboratory for resistance to high temperature and relative contents of three stress proteins (Hsp-70, artemin and p26). Thermal adaptation took place rapidly, during the first growing season. The increase in thermal tolerance was reflected in an overall increase in stress protein content, compared to SFB cysts used for the initial inoculation. Also examined were cysts of *A. tibetiana* collected from a lake on the high plateau of Tibet, PR China, almost 4.5 km above sea level. These cysts were very sensitive to high temperatures, and contained much lower levels of all stress proteins examined, compared to *A. franciscana* cysts from SFB and Vietnam. Cysts of *A. sinica*, collected from a hypersaline lake in Inner Mongolia, PR China, were examined in the same fashion and found to be similar to SFB cysts in terms of thermal resistance and stress protein content. The harsh environments in which *Artemia* are found, and the great diversity of its habitats, world-wide, provide excellent opportunities to relate the ecological setting of an organism to the underlying physiological and biochemical processes enabling its survival.

### Introduction

Species of the brine shrimp, *Artemia*, are found in a variety of harsh environments world wide (Triantaphyllidis et al., 1998) in which they encounter severe hypersalinity, high doses of ultraviolet radiation, very low oxygen tensions and extremes of temperature (Clegg & Conte, 1980; Persoone et al., 1980; Decleir et al., 1987; Warner et al., 1989; MacRae et al., 1989; Browne et al., 1991; Hand & Hardewig, 1996). These challenging ecological settings suggest that *Artemia* represents a useful model organism for studies on the stress response at all levels of biological organization. The stress (heat shock) response has been the object of study so intense that the volume of literature is overwhelming (selected reviews and

books are cited as an entry – Nover, 1991; Parsell & Lindquist, 1993; Morimoto et al., 1994; Fiege et al., 1996; Schöffl et al., 1998; Fink & Golo, 1998; Feder & Hofmann, 1999). One feature of the heat shock response is 'induced thermotolerance' (ITT), the ability of a sub-lethal heat shock, well above physiological temperature (but below lethal) to induce resistance to a subsequent, otherwise lethal heat exposure. The basis for ITT is not understood completely, but it has been well-documented that a major contribution comes from an ensemble of heat shock (stress) protein families, many of which act as molecular chaperones to minimize aggregation of unfolded globular proteins and, in some cases, to restore native structures. Once again, the literature is massive (see entry references above, and Beissinger & Buchner, 1998; Lorimer &

Baldwin, 1998; Richardson et al., 1998; Ellis, 1999; Ellis & Hartl, 1999; Fink, 1999; Houry et al., 1999; Van den IJssel et al., 1999; Whitely et al., 1999). Interestingly, thermotolerance has not been demonstrated in *Artemia* cysts in spite of several attempts (Miller & McLennan, 1988a; Liang & MacRae, 1999; Clegg et al., 1999) while ITT is readily demonstrated in larvae (Miller & McLennan, 1988a) and adults (Frankenberg & Clegg, 2000). We will comment on the possible significance of those observations later in this paper.

Current research on the stress response is aimed chiefly at understanding the cellular and molecular basis for heat shock protein structure and function. As pointed out in the excellent review by Feder & Hofmann (1999), comparatively little research has been devoted to the evolutionary and ecological aspects, although these topics have been given increased recent attention (see Feder & Block, 1991; Coleman et al., 1995; Norris et al., 1995; Somero, 1995; Hightower, 1995; Clegg et al., 1999; Krebs, 1999; Tomanek & Somero, 1999). We believe that *Artemia* can be used to great advantage in that regard, a belief that motivated the present studies.

Most experimental research on *Artemia* sp. has used *A. franciscana* which occurs naturally, and primarily in the Great Salt Lake, Utah, and in salt-erns located in the San Francisco Bay (SFB), although many other populations exist, due chiefly to human activity. In 1983, cysts from the SFB were first inoculated into ponds in central Vietnam (Quynh & Lam, 1987). Since 1987, *Artemia* has been grown on a seasonal basis during the dry season in southern Vietnam (Baert et al., 1997). Because ambient temperatures are much higher in these ponds, compared to SFB salt-erns, it seemed worthwhile to compare cysts from SFB with those produced in Vietnam to determine whether the latter have acquired enhanced resistance to higher temperatures and, if so, to evaluate how rapidly that adaptation took place. Results presented here on this comparison represent the first from an ongoing research project in Vietnam, and the study of other *Artemia* species from widely different thermal habitats around the world (Triantaphyllidis et al., 1998). In the present case, we compare the thermal tolerance and stress proteins of cysts produced by sequential inoculation of SFB cysts in the Vietnam culture ponds during the years 1996, 1997 and 1998. In addition, the thermal responses of cysts of two other species, *A. sinica* from Inner Mongolia, and *A. tibetiana* from the Tibet high plateau, have been examined similarly.

## Materials and methods

### *Origins of cysts and hatching assays*

*A. franciscana* cysts from the South San Francisco Bay (SFB) U.S.A. were obtained from the *Artemia* Reference Center, Ghent University, Belgium, as part of the cyst collection used for the International Study of *Artemia* (ISA). These cysts (ISA 1258) were used to inoculate growth ponds in Vietnam in the dry season of 1996, and cysts produced by the resulting adults were used for inoculation in 1997; likewise, cysts from that year provided the inoculum for the 1998 growing season. These cysts will be referred to as Y1, Y2 and Y3, respectively. Thus, we obtained cysts from animals that had experienced one, two or three seasons of growth and reproduction in these ponds. This project, referred to here as 'the Vietnam experiment', was carried out in experimental ponds of the Institute of Marine Aquaculture, Can Tho University, located in the salt-erns of the Vinh Tien Shrimp-Salt Cooperative, Vin Chau District, in the Mekong Delta of Vietnam (see Baert et al., 1997). Salinity ranges (ppt) in the growth ponds were 62–115 for Y1, 75–87 for Y2 and 74–84 for Y3. The daily water temperatures during the growing season in all 3 years ranged from 24 to 38 °C, essentially on a daily basis. Cysts were harvested over roughly the same period during the growing seasons in each of the 3 years.

Cyst viability was determined using 20-well plastic depression plates, each well containing 10–30 embryos in 400 µl of sea water (SW). The plates were covered, sealed with tape to prevent evaporation, and incubated in constant light at 22±1 °C until hatching was complete. For each group of cysts 4 such plates were used, each containing at least 200 cysts.

*A. sinica* cysts were provided by the Salt Research Institute, Tanggu, PR China, and cysts of *A. tibetiana* were obtained from Professor Zheng Mianping, Chinese Academy of Geological Sciences, Beijing, PR China. *A. sinica* cysts were collected from a hypersaline, carbonate lake near Yimeng (160 ppt salinity) located in Inner Mongolia (108° 55' E, 39° 10' N) at an elevation of about 1300 m. *A. tibetiana* cysts were obtained from Lagkor Lake, also hypersaline (91 ppt) and carbonate, located on the high plateau of Tibet (84° E, 32° 03' N) at 4490 m elevation where the average yearly water temperature is 1–2 °C. Further details on this new species and its location have been published (Abatzopoulos et al., 1998; Zunyng et al., 1998).

### Heat shock (HS) and protein preparation

Dried cysts were hydrated in SW at 2 °C for 16–18 h, collected on cloth filters, and washed briefly with distilled water (10 s) followed by blotting on paper towels for 2 min to remove interstitial water. These cysts were either used as unheated controls or transferred to 50 ml centrifuge tubes containing 35 ml of SW preheated to 22.0 °C in a Lauda RM water bath good to  $\pm 0.05$  °C. The temperature was then increased to 50.0 °C at a rate of 0.7 degrees  $\text{min}^{-1}$  with continuous vigorous aeration. This protocol was used to avoid transfer of cysts directly from 2 °C to a much higher temperature. Temperatures were measured in the incubation tubes using a precision thermometer ( $\pm 0.05$  °C). Cysts were held at 50.0 °C for various times, collected as described above, assayed for viability and, in some cases, prepared for stress protein analysis. In the latter case, cysts were homogenized on ice at 100 mg wet weight  $\text{ml}^{-1}$  of buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM  $\text{MgCl}_2$ , 5 mM  $\text{NaH}_2\text{PO}_4$ , 40 mM Hepes, pH 7.4). Homogenate aliquots were combined separately with equal volumes of 2 $\times$ SDS sample buffer, vortexed and heated at 100 °C for 5 min (Laemmli, 1970). After cooling, the preparations were centrifuged (1630 g, 3 min) to remove insoluble fragments of the chitinous shells that prevent accurate pipetting. Aliquots equivalent to the same wet weight of cysts were applied to the gels to enable direct comparison between samples. That basis seems better than applying the same amounts of protein because of the domination of yolk platelet proteins in these embryos.

### SDS-PAGE and Western immunoblotting

SDS-preparations were electrophoresed on 12% polyacrylamide gels. For each experiment, two identical gels were run: one was stained with Coomassie Blue [500 mg Serva Blue 500  $\text{ml}^{-1}$  of methanol:acetic acid: $\text{H}_2\text{O}$  (40:50:10)] and proteins on the other were transferred to a nitrocellulose membrane (Towbin et al., 1979) for Western immunoblot analysis. Membranes were incubated with monoclonal anti-hsp-70 antibody, clone 7.10 (Affinity BioReagents, Inc., Golden, Colorado). Goat anti-rat IgG conjugated with horseradish peroxidase was used for the secondary antibody (Southern Biotechnology Associates, Inc., Birmingham, Alabama). ECL Western Blotting Reagents (Amersham Corp., Arlington Heights, Illinois) were used for detection of labeled proteins on Kodak BioMax MR single emulsion film (Eastman Kodak Co., Rochester, New York) and developed in an X-Ray

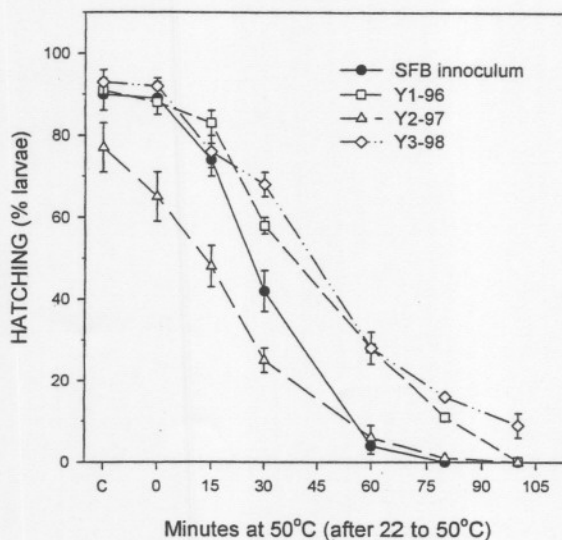


Figure 1. Hatching levels of cysts from the Vietnam experiment following return to  $22 \pm 1$  °C after exposure to 50.0 °C for the times shown. Unheated cysts are controls (C) and Y1, Y2, Y3 refer to cysts produced during the growing seasons of 1996, 1997, 1998, respectively. See text for further details.

film processor. Similar protocols were used to detect artemin, a ferritin-like stress protein present in cysts (De Herdt et al., 1980; De Graaf et al., 1990) and p26, a small heat shock/ $\alpha$ -crystallin protein of known importance to cyst stress resistance (see Clegg et al., 1999; Liang & MacRae, 1999). Differences from the Western blotting described above for Hsp-70 involved the use of anti-artemin, provided by Herman Slegers, and our anti-p26, both being polyclonal and affinity-purified before use (see Clegg et al., 1994, 1999 for further details).

## Results

### The Vietnam experiment

Figure 1 describes the effects of high temperature on viability (% hatching) of the SFB cysts used for the inoculation in 1996, and on cysts produced in Vietnam during the three growth seasons of the study (1996–1998). No differences in hatching performance were noted for cysts produced in Vietnam during 1996 and 1998, compared to the SFB inoculum, during the initial 15 min exposure to 50.0 °C. As exposure time at that temperature increased, the 1996 and 1998 cysts proved to be much more resistant than the SFB inoculum. In contrast, hatching percentages of the cysts



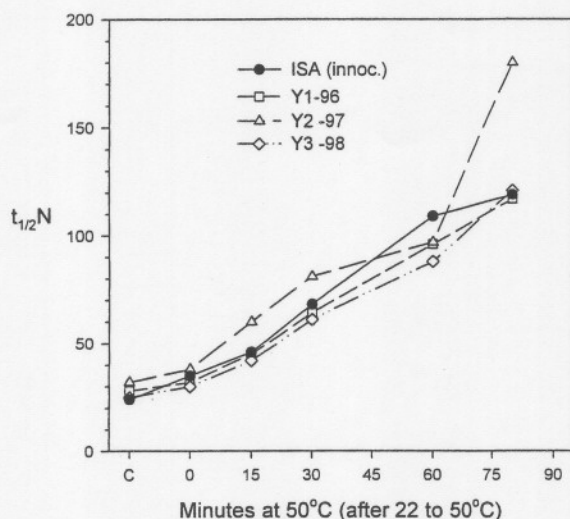


Figure 2. Developmental rates of cysts previously exposed to the heating conditions described in Figure 1. The time in hours required to produce one-half the final hatching percentage is referred to as  $t_{1/2}N$ .

produced in 1997 were lower than all the others, and their thermal resistance much poorer (Fig. 1). Even when the 1997 data were normalized to compensate for the lower hatching levels of unheated controls, the trends seen in Figure 1 remained the same (not shown). The poor performance of the 1997 crop was, unfortunately, the result of improper storage conditions in Vietnam after harvest in 1997 and before the cysts were shipped to Belgium and assayed during the winter of 1999. Therefore, we place no weight on results obtained using these defective cysts.

Figure 2 describes the developmental rate of the cysts used in the heating study (Fig. 1), measured by the time in hours required for 50% of the final hatching level to be achieved ( $t_{1/2}N$ ). The delays in development as a function of heating exposure time were similar for all cysts with the exception of those produced in 1997, another indication of their poor quality.

#### Thermal performance of *A. sinica* and *A. tibetiana* cysts

The response of these cysts to the same heating protocol used above is shown in Figure 3, where they are compared to the SFB inoculum (ISA) and to cysts produced in Vietnam (Y3-98). In this case the hatching levels were normalized to unheated controls to facilitate comparison: actual control hatching percentages were  $79.7 \pm 2.2\%$  for *A. sinica*, and  $79.0 \pm 3.3\%$  for

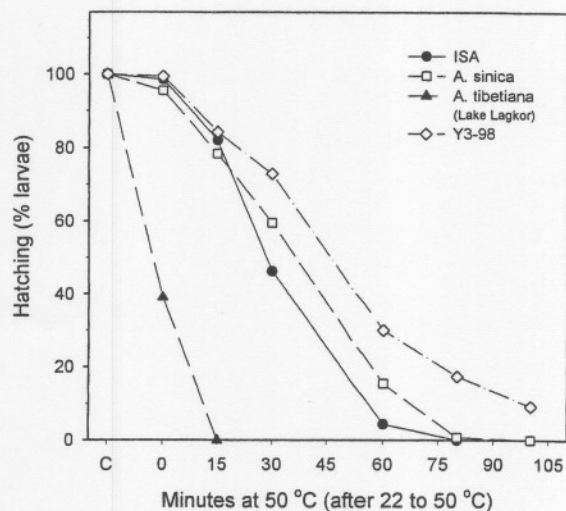


Figure 3. Responses of cysts of different species of *Artemia* to the same heating regime used in the study described in Figure 1. Hatching levels are normalized to the unheated controls, set to 100%.

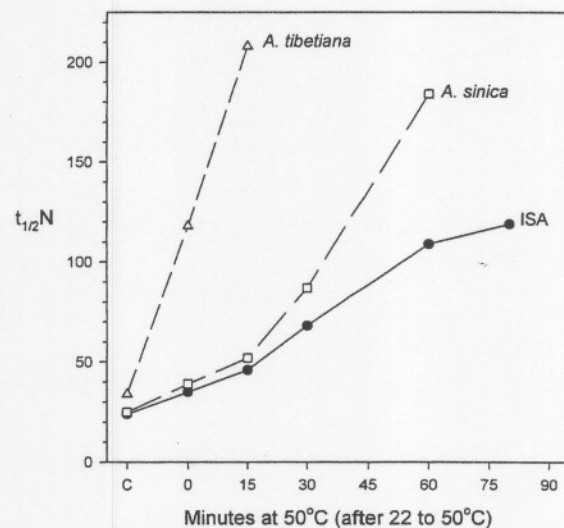


Figure 4. Developmental rates ( $t_{1/2}N$ , in hours) of the cysts studied in Figure 3. ISA (International Study on *Artemia*) refers to *A. franciscana* cysts produced in the South San Francisco Bay, California.

*A. tibetiana* (means  $\pm$  standard errors,  $n=4$  plates of at least 200 cysts each). Standard errors associated with these measurements were similar in extent to those shown in Figure 1.

The thermal lability of *A. tibetiana* is obvious, over half of them being killed during the 40 min transition from 22.0 to 50.0 °C. The performance of *A. sinica* cysts was comparable to both groups of *A. franciscana* cysts during the initial 15 min of heating at 50.0 °C,

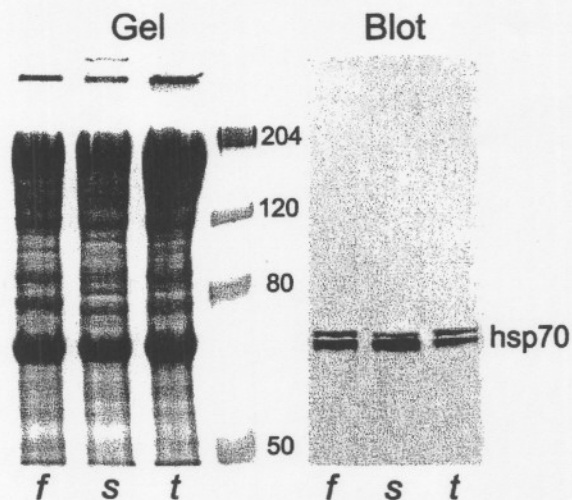


Figure 5. Electrophoretic profiles of proteins in cysts of ISA *A. franciscana* (f), *A. sinica* (s) and *A. tibetiana* (t). 'Gel' shows Coomassie-stained proteins, and 'Blot' describes the corresponding Western immunoblot for the Hsp-70 family. Numbers in the center column refer to molecular masses of protein standards (kilodaltons).

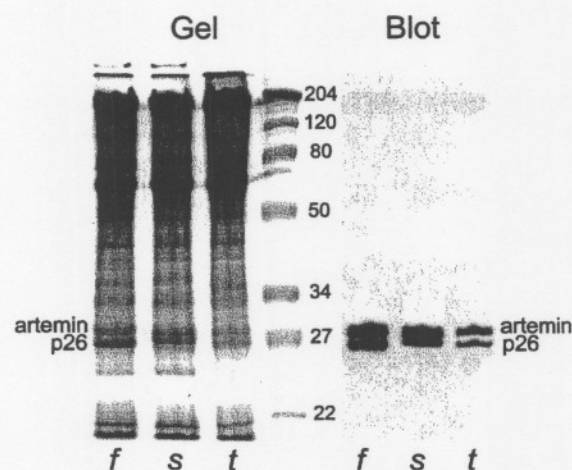


Figure 6. Protein profiles and Western blot detection of artemin and p26. See caption to Figure 5 and 'Materials and methods' for further details.

then became intermediate until all were killed after 80 min. Developmental rates ( $t_{1/2N}$ , in hours) for cysts of these two species are compared with those for *A. franciscana* (ISA) in Figure 4.

#### Stress (heat shock) proteins

Profiles of total proteins and two isoforms of the Hsp-70 family in cysts of *A. franciscana* (f), *A. sinica* (s) and *A. tibetiana* (t) are shown in Figure 5. Coomassie-stained proteins are shown to the left (Gel)

and Hsp-70 detected by Western immunoblotting to the right (Blot). All three populations of cysts contained two isoforms of slightly different molecular mass, but cysts from Tibet contained less of both isoforms, notably since these cysts also contain slightly more protein per unit wet cyst weight than the other cyst samples as can be seen in the Gel in Figure 5 (and unpublished results). Comparable results for the ferritin-like protein artemin, and the small heat shock/ $\alpha$ -crystallin protein, p26, are shown in Figure 6. For both stress proteins, the abundance is clearly *franciscana* > *sinica* >> *tibetiana*. In addition, the molecular masses of p26 in the latter two cyst samples seem to be slightly lower than p26 from *A. franciscana* cysts, although that requires further study.

Relative amounts of these four proteins have been estimated by scanning of Western blots followed by densitometry (Table 1). In addition to the three cyst species examined above, we include comparable results from cysts produced in 1998 in Vietnam, the most heat-resistant of all cysts described in the present paper (Y3-98, Fig. 1). These results have been normalized to the levels of these proteins in the 1998 Vietnamese cysts, shown as 100% in Table 1. Comparisons can be made between different cyst groups for the same protein since all these samples were examined on the same blot, but comparisons between the different proteins are not valid. The thermal lability of *A. tibetiana* cysts is mirrored in their relatively low content of all these stress proteins. Similarly, the 1998 Vietnamese cysts contain more artemin and p26 than cysts of the other groups, and that is particularly interesting in comparison to the ISA inoculum from which they were derived. On the other hand, the 1998 cysts contained relatively smaller amounts of the Hsp-70 family.

#### Discussion

As mentioned, encysted embryos from the SFB population (ISA) were used to establish field cultures in southern Vietnam where water temperatures were substantially higher than those of the San Francisco Bay. Maximum water temperatures in the latter during the Summer and Fall growing season very rarely exceed 24 °C, and typically are several degrees lower (Robert Rofen, pers. comm.). In contrast, the Vietnamese populations experienced daily water temperatures near 38 °C for most of the growing seasons and never below 24 °C (Peter Baert, pers. comm.; Baert et al.,

Table 1. Comparison of the relative amounts of stress (heat shock) proteins in *Artemia* cysts of different species and from different locations

Cyst origin <sup>a</sup>	Relative amount as a percentage of the Y3-98 cyst content			
	p26	artemin	Hsp-70 <sub>high</sub>	Hsp-70 <sub>low</sub>
Y3-98	100	100	100	100
ISA/SFB	65	86	123	49
<i>A. sinica</i>	76	49	111	226
<i>A. tibetiana</i>	9	23	45	51

<sup>a</sup>ISA/SFB are *A. franciscana* cysts used for the initial inoculation in the Vietnam experiment, and Y3-98 are cysts produced in those ponds during the third sequential growing season. Details are given in 'Materials and methods' for *A. sinica* and *A. tibetiana*.

1997). Also recall that the culture of these animals was done on a seasonal basis: cysts produced during the preceding growing season were used to establish, by cyst inoculation, animals produced during the next year's growing season. This 'sequential' procedure should favor the accumulation of adaptations leading to increased thermal resistance of cysts produced in Vietnam.

To examine that possibility the Vietnam experiment was initiated during the growing season of 1996, and continued in 1997 and 1998. Except for the 1997 cysts, which had been stored improperly, the results (Fig. 1) showed that thermal resistance of Vietnamese cysts was indeed better than the SFB cysts used for the inoculation, even after a single growing season. That finding agrees with the detailed study by Browne & Wanigasekera (2000) who found that SFB *A. franciscana* showed the highest degree of phenotypic plasticity of the five *Artemia* species examined in the laboratory. Interestingly, they also concluded that the upper thermal limit for successful reproduction of SFB animals was close to 30 °C, providing further reason to believe that animals grown in the Vietnam ponds must have acquired enhanced thermal tolerance in the first generation.

Although most of that improvement took place during the first growing season (Fig. 1, Y1-96) it appears that those produced during 1998 are more resistant to longer exposures at 50.0 °C. These results at the level of intact encysted embryos can be compared to the levels of their stress proteins (Figs 5 and 6; Table 1). Cysts produced in Vietnam contained substantially higher levels of artemin, p26 and the low molecular weight isoform of Hsp-70 than did the cysts used for the inoculation. The Hsp-70 family of stress proteins is well known to play a major role in coping with damage caused by high temperatures and other stressors, in a wide variety of cells and organisms (Bukau & Horwich, 1998; Karlin & Brocchieri,

1998; Kiang & Tsokos, 1998; Krebs, 1999; Nollen et al., 1999). Most research on this Hsp-family has focused on cellular and molecular aspects, but there is growing interest in the organismic and ecological levels of organization (reviewed by Feder & Hofmann, 1999). More recent examples are studies by Krebs (1999) on Hsp-70 in larvae and adults of three species of *Drosophila* from different thermal environments, and by Tomanek & Somero (1999) on marine snails from different locations in the intertidal. Both of these excellent studies illustrate the difficulty of uncovering causal connections between levels of stress proteins, thermotolerance and ecological setting.

Our results on cyst Hsp-70 using 1-D gels are similar to those obtained by Miller & McLennan (1980b) who carried out a much more detailed study of cyst and larval stress proteins, demonstrating several isoforms of the Hsp-70 and -90 families, some of which were induced by heat shock. Using radioactive labeling, they also observed synthesis of a small stress protein that might be artemin, although the molecular mass they reported is higher by about 3 kilodaltons. The function of artemin is not known (De Herdt et al., 1979; De Graaf et al., 1990) but it appears to be a stress protein since its synthesis is strongly upregulated following prolonged anoxia, which suppresses protein synthesis in general (Clegg & Jackson, 1998), and its primary structure bears some resemblance to another stress protein, ferritin (De Graaf et al., 1990). In contrast, previous work has documented the importance of p26 in the stress resistance of cysts: they contain massive amounts of this small heat shock/ $\alpha$ -crystallin protein which undergo extensive stress-induced translocations from the 'soluble' phase of cell extracts to nuclei and other cell compartments (Clegg et al., 1994, 1995, 1999; Jackson & Clegg, 1996). P26 exhibits substantial molecular chaperone activity *in vitro* (Liang et al., 1997a,b) and probably functions that way *in vivo* (Liang & MacRae, 1999). We believe the in-



crease in thermal resistance of the Vietnamese cysts compared to those of the ISA inoculum (Fig. 1) involves elevated levels of this important protein (Table 1). However, that is probably not the whole story since *Artemia* cysts also contain very large amounts of the compatible solutes, trehalose and glycerol (see Clegg & Conte, 1980) well known for their stabilizing properties (see Yancey et al., 1983; Crowe et al., 1992, 1996; Winzor et al., 1992). Finally, we recognize that differences other than temperature exist between the San Francisco Bay and the growth ponds in Vietnam. But there is no way of knowing the extent to which those uncontrolled variables are involved, if at all, in the different performances of these cysts during exposure to high temperatures. It seems likely that animals in the growth ponds are responding to stresses in addition to temperature, salinity being a probable one (Browne & Wanigsekera, 2000).

Considering the very cold habitat of *A. tibetiana*, it is not surprising that their cysts do not survive very long at high temperatures (Fig. 3). Once again, there seems to be good agreement between poor cyst survival under these conditions and low levels of stress proteins, compared to their amounts in the other cysts (Table 1). Protein synthesis is an expensive process, so natural selection might have favored a reduction in constitutive levels of these proteins which are probably less important to these animals in view of their very cold environment. *A. sinica* cysts perform slightly better than the SFB population, at least under the thermal regime used here (Fig. 2) and, although they contain less artemin, they have substantially more p26 and much more of the low molecular mass Hsp-70 isoform compared to SFB cysts. In the absence of more details about the habitat of these animals, little more can be said. However, if we suppose that responses of cysts to high temperatures in the laboratory, and the levels of their stress proteins are 'predictive' in terms of the thermal habitat in which they were produced, then we speculate that SFB *A. franciscana* and *A. sinica* live under approximately the same thermal conditions.

Delays in the rate of embryonic development following bouts of stress (Figs 2 and 4) have been observed before, for anoxia (see Clegg, 1997) and heat shock (Miller & McLennan, 1988a; Clegg & Jackson, 1992). What causes these delays? An obvious possibility would be the need to repair damage resulting from that stress. Less obvious, but more interesting, is the possibility that the mechanisms protecting the embryo during stress require time to be reversed, and the time needed for that being a function of the severity and/or

duration of the previously-experienced stress. Some support for that possibility comes from the observation that anoxia and heat shock force at least some of the exposed cysts to re-enter a diapause-like condition in which they remain, even when conditions conducive to development are restored (Clegg & Jackson, 1992; Abatzopoulos et al., 1994; Clegg, 1997). We should point out that the greater portion of these post-stress delays takes place during development of the encysted embryo, prior to its emergence from the shell (Clegg, 1997) a point that is not obvious from the parameter,  $t_{1/2N}$  (Figs 2 and 4). Thus, whatever causes the post-stress delay it has largely been removed by the time that emergence and hatching occur.

We return to the results of Miller & McLennan (1988a) on encysted embryos of *A. franciscana* from the Great Salt Lake, Utah, and larvae hatched from them in the laboratory. As expected, they found that cysts were quite tolerant to heating, the  $LT_{50}$  being close to 49 °C (using 1 h exposures). Larvae 24 h after hatching from these cysts were much less tolerant, with an  $LT_{50}$  of 42 °C (1 h exposures). What is the basis for these differences between cysts and larvae? Trehalose, glycerol, artemin and p26 are present in greatly reduced amounts in newly hatched larvae, and disappear during the first instar. Other differences of potential importance include the absence of DNA synthesis and cell division in encysted embryos (reviewed by Clegg & Conte, 1980; also see Miller & McLennan, 1988a,b) both of which occur in larvae. Another factor concerns the greater tissue complexity of larvae compared to encysted embryos. Larvae obtained from cysts of the Vietnam experiment have yet to be examined for resistance to high temperatures, and such a study should be interesting.

Curiously, although encysted embryos synthesize stress proteins, they do not appear to be capable of induced thermotolerance (ITT), as reported by Miller & McLennan (1988a). Other attempts to demonstrate ITT have resulted in the same outcome (Liang & MacRae, 1999; Clegg et al., 1999). In contrast, ITT is readily demonstrated in other stages of *Artemia*'s life cycle (Miller & McLennan, 1988a; Frankenberg et al., 2000). Indeed, with very rare exception ITT is the rule in the heat shock responses of a wide variety of organisms (Nover, 1991). The lack of ITT in cysts might be due to the establishment during the formation of the encysted embryo of a thermal tolerance that cannot be improved upon (see Clegg et al., 1999). Alternatively, this aspect of the heat shock response might be suppressed since the metabolism of

these embryos is based on endogenous substrates, and strictly limited in terms of amino acid precursors and energy substrates that might be needed for embryonic development (Clegg & Conte, 1980).

Because of the widely different protocols used for heat shock studies (time/temperature) and the often unstated thermal characteristics of the natural setting of the organisms examined, it is difficult to compare our results on *Artemia* with findings on other invertebrates, at least in the context of adaptation. In this regard, Nover (1991) compiled heat shock response data in 15 pages of tables which illustrate this experimental variation, and emphasize the widely different ecological settings of the organisms under study, both of which make it difficult to extract generalities. Be that as it may, we believe the results described in this paper document further the utility of *Artemia* as a model for the study of biochemical adaptation.

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